ABSTRACT

Background: Benincasa hispida (Thunb.) Cogn. is an extensive climbing annual herb in an agricultural country like India. Lupeol, a constituent of this species, has been reported to possess good amount of pharmacological potential. Objectives: In the current studies, the research team focused on determining the percentage of the lupeol present in the extract of Benincasa hispida seeds by chromatographic techniques. Materials and Methods: Shade-dried seeds of Benincasa hispida were subjected to soxhlet extraction followed by scrutinization of the lupeol contents by HPTLC and HPLC methods after carrying out preliminary phytochemical screening for the constituents present in the extract. Results: The extraction yield was found to be 1.2% (w/w). Phytochemical screening of the extract revealed the presence of carbohydrates, glycosides, alkaloids, fixed oils and fats, tannins phenolic compounds, steroids and flavonoids. The amount of lupeol present in the seeds extract was found to be 0.47% w/w (HPTLC) and 6.85% w/v (HPLC) by HPTLC quantification and HPLC analysis respectively. Research studies showed a peak which coincided with the peak of standard lupeol signifying the presence of lupeol in the extract. Conclusion: The extract contains significant amount of lupeol.

Keywords: Benincasa hispida, Soxhlet extraction, HPLC, HPTLC, lupeol

INTRODUCTION

India has one of the richest medicinal plant traditions in the world with remarkable contemporary relevance for ensuring health security to millions. Around 25,000 effective plant-based formulations and folk medicines are known to rural communities that are used for medicinal purposes in preventive and curative applications.[1] We have selected Benincasa hispida (Thunb.) Cogn. species for our research studies which is found to be cultivated throughout the plains of India, Burma and Ceylon on hills upto 4000ft. In addition, it is described as best fruit among all Valliphala. The classical medicines reported from the selected plant are Kushmanda avaleha, Vasakhanda, Khanda and Rasayana.[2–4] Due to its rasayana property, it is beneficial in improving immune protection and is advised during the degenerative phase of life around 45 years in both the sexes.[5] The fruit contains good amount of proteins, enzymes, vitamin B1 and C, flavonoid C-glycoside, terpenes, phenolic acids and free sugars such as glucose, rhamnose, mannitol, uronic acid and some trace metals which are beneficial in treatment of various diseases such as diabetes, cancer, inflammatory disorders, convulsions and infections. Pectic polysaccharides have been obtained from the fruits by sequential extraction[6,7] while three phenolic compounds viz. astilbin, catechin and naringenin by high-speed counter current chromatography.[8] Bioactive proteins were isolated and characterized from each of the parts and the highest yield was reported in roots.[9]

Taking into considerations the literature citations, our research article attempts to find out how much percentage of the lupeol is present in the Benincasa hispida seeds extract which is reported for having a number of important bioactivities such as antiarthritic, antiproto-
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zal, anti-inflammatory, anticancer, hepatoprotective and chemoprevention.\[^{10,11}\] Though literature reports several methods for quantification of lupeol from various plants, no studies on bioactivity guided fractionation for isolation and quantification of lupeol from *Benincasa hispida* seeds extract has been reported.

**MATERIALS AND METHODS**

**Part A: Collection, authentication and extraction**

Fresh seeds of *Benincasa hispida* were collected from Mumbai local market in the month of April-May and shade-dried. They were authenticated by Agharkar Research Institute, Pune. A voucher specimen (No.3/187/2013/Adm.1692/083) was deposited in the botany department of Agharkar Research Institute, Pune. The seeds were subjected to a soxhlet extraction procedure as follows:

1. Step 1: 31 gm of seed powder was packed in soxhlet thimble.
2. Step 2: 300 ml petroleum ether was taken in round bottom flask.
3. Step 3: The sample was extracted until the solvent in soxhlet thimble became colorless.
4. Step 4: The concentrate was evaporated to dryness under reduced pressure at 40°C using rotary evaporator.
5. Step 5: The extract was collected and stored in an airtight amber colored glass container.

This petroleum ether extract of *Benincasa hispida* seeds was subjected to analytical studies by comparison with standard biomarkers after carrying out the preliminary qualitative phytochemical screening.\[^{12–14}\] All the standard biomarkers used for identification purpose in analytical studies were obtained from Sigma-Aldrich Private Limited, India, and solvents from Merck, India, including HPLC grades. Separation and identification of active components from the seed extract formed the basis for HPTLC and HPLC methods. These are sophisticated powerful visualization techniques which are preferred in the detection of the constituents in the extracts due to its accuracy, preciseness, specificity, sensitivity and reproducibility.\[^{15}\]

**Part B: Analytical studies**

Our research studies encompasses Thin Layer Chromatography (TLC), High Performance Thin Layer Chromatography (HPTLC) and High Pressure Liquid Chromatography (HPLC) for determining the percentage of lupeol present as an active constituent in the selected seed extract of BH plant species.

**a) Thin Layer Chromatography (TLC)**

Mobile Phase: Toluene: Ethyl Acetate (9.5:0.5) was used for the study. The standard and the sample were dissolved in ethanol and was filtered using Whatman Filter paper no. 41. The TLC chamber was saturated for 30 mins.

**b) High Performance Thin Layer Chromatography (HPTLC)**

The HPTLC was performed at Radiant Research Laboratories Private Limited, Bangalore. The analysis was carried out by application of the sample and the standard dissolved in methanol on HPTLC plates (20 × 10 cm) coated with silica gel 60 F254. Scanning of the developed plates was carried out at 333nm and 550nm. The standard and the sample were prepared by dissolving 5.16mg and 47.5mg in 10ml of solvent each. Spots of 3 µg/l, 6 µg/l, 9 µg/l and 12 µg/l were applied on HPTLC plates. Instrument used was CAMAG Linomat 5 with spray gas as an inert gas, sample solvent as methanol, dosage speed of 150 nl/s and predosage volume: 0.2 µl. The HPTLC details comprises of syringe size of 100 µl. Ten tracks with application position of 12.0 mm and band length of 8.0 mm were used. Calibration mode of single level, statistics mode with CV and evaluation with peak height and area (percentage) were the analyzed parameters.

Formula: Percentage of lupeol = sample area × standard dilution × purity × 100/standard area × sample dilution × 100

**c) High Pressure Liquid Chromatography (HPLC) analysis**

HPLC instrument used was Shimadzu LC-10 ATVP with software as Chromtech N 2000 data with a detector of 280 nm and a flow rate of 1.5 ml/min. The injection volume was 20µl and column dimensions were RP C-18, 250 × 4.6 mm, 5 µ. Mobile phase used was acetonitrile and water (95:5). 100µg of both, the standard and sample were dissolved in 1 ml of the solvent. From this stock solution, 20 µl was injected for experimental purpose.

Formula: Percentage of lupeol = sample area × standard dilution × purity × 100/standard area × sample dilution × 100.
RESULTS

(a) Extraction yield

The extraction yield was found to be 1.2% w/w (petroleum ether extract).

(b) Preliminary analysis of the plant extracts

The petroleum ether extract was been found to be positive for carbohydrates, glycosides, alkaloids, fixed oils and fats, tannins, phenolic compounds, steroids and flavonoids. (Table 1).

(c) Chromatographic analysis of extracts

(i) TLC reports

Rf value for standard lupeol and the extract was found to be 0.21 which confirms the presence of the active constituent in the seed extract (Figure 1).

(ii) HPTLC reports

The seed extract showed well resolved spots at tracks 5 and 6 in comparison to the standard at tracks 1, 2, 3, 4, 9, 10, 11 and 12. The images were obtained at 333 nm and 550 nm before and after derivatization respectively. The Rf value was found to be equal with lupeol [(Start 0.50, maximum 0.56 and end 0.57)] (Table 2 and Figures 2–11). The amount of lupeol present in the extract was 0.47 % w/w (i.e. 0.22 mg of lupeol present in 47.5 mg of extract).

Table 1. Preliminary qualitative phytochemical analysis of the plant extract

<table>
<thead>
<tr>
<th>Test for</th>
<th>Reagent</th>
<th>Observation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbohydrates</td>
<td>Molish's reagent</td>
<td>Present</td>
</tr>
<tr>
<td>Reducing sugars</td>
<td>Fehling's reagent</td>
<td>Present</td>
</tr>
<tr>
<td>Saponin glycosides</td>
<td>Formation of Foam</td>
<td>Present</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>Shinoda reagent</td>
<td>Present</td>
</tr>
<tr>
<td>Alkaloids</td>
<td>Dragendorff’s reagent</td>
<td>Present</td>
</tr>
<tr>
<td>Tannins and Phenolic compounds</td>
<td>5% FeCl3 solution</td>
<td>Present</td>
</tr>
<tr>
<td>Mucilage with powdered drug material</td>
<td>Ruthenium red</td>
<td>Absent</td>
</tr>
<tr>
<td>Steroids</td>
<td>Salkowski reagent</td>
<td>Present</td>
</tr>
<tr>
<td>Fats and Oils</td>
<td>Sudan Red III reagent</td>
<td>Present</td>
</tr>
</tbody>
</table>

Table 2. HPTLC analysis of Benincasa hispida and standard lupeol

<table>
<thead>
<tr>
<th>Track No</th>
<th>Details</th>
<th>Height</th>
<th>Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>lupeol (3µg/l)</td>
<td>146.3</td>
<td>4123.5</td>
</tr>
<tr>
<td>2</td>
<td>lupeol (6µg/l)</td>
<td>265.6</td>
<td>7144.5</td>
</tr>
<tr>
<td>3</td>
<td>lupeol (9µg/l)</td>
<td>332.6</td>
<td>9307.5</td>
</tr>
<tr>
<td>4</td>
<td>lupeol (12µg/l)</td>
<td>384.1</td>
<td>11146.7</td>
</tr>
<tr>
<td>5</td>
<td>Benincasa hispida</td>
<td>12.4</td>
<td>330.3</td>
</tr>
<tr>
<td></td>
<td>petroleum ether extract</td>
<td>125.1</td>
<td>3061.5</td>
</tr>
<tr>
<td>6</td>
<td>Benincasa hispida</td>
<td>125.1</td>
<td>3061.5</td>
</tr>
<tr>
<td></td>
<td>petroleum ether extract</td>
<td>125.1</td>
<td>3061.5</td>
</tr>
<tr>
<td>9</td>
<td>lupeol (12µg/l)</td>
<td>370.9</td>
<td>10527.7</td>
</tr>
<tr>
<td>10</td>
<td>lupeol (9µg/l)</td>
<td>313.2</td>
<td>8656.6</td>
</tr>
<tr>
<td>11</td>
<td>lupeol (6 µg/l)</td>
<td>239.2</td>
<td>6272.9</td>
</tr>
<tr>
<td>12</td>
<td>lupeol (3µg/l)</td>
<td>130.6</td>
<td>3500.4</td>
</tr>
</tbody>
</table>

Figure 1. TLC of Benincasa hispida.

Figure 2. Track 1 - HPTLC peak of standard Lupeol.
Figure 3. Track 2 - HPTLC peak of standard lupeol.

Figure 4. Track 3 - HPTLC peak of standard lupeol.

Figure 5. Track 4 - HPTLC peak of standard lupeol.

Figure 6. Track 9 HPTLC peak of standard lupeol.

Figure 7. Track 10 - HPTLC peak of standard lupeol.

Figure 8. Track 11 - HPTLC peak of standard lupeol.
Figure 9. Track 12 - HPTLC peak of standard lupeol.

Figure 10. Track 5 - HPTLC peak of Benincasa hispida seeds extract.

Figure 11: Track 6 - HPTLC peak of Benincasa hispida seeds extract.

Figure 12. HPTLC Image before Derivatization.

Figure 13. HPTLC Image after Derivatization.
(iii) HPLC reports

The petroleum ether BH seeds extract showed a characteristic retention peak (15.332 min) at 280 nm confirming the presence of lupeol at a flow rate of 1 ml/min using methanol solvent system (Table 3, Figures 14–15). The amount of lupeol present was 6.85% w/v.

Table 3. HPLC analysis of Benincasa hispida and standard lupeol

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Retention) Sample area of lupeol</td>
<td>(15.237) 9498.441</td>
</tr>
<tr>
<td>(Retention) Standard area of lupeol</td>
<td>(15.532) 124739.67</td>
</tr>
<tr>
<td>Dilution of lupeol (Sample and Standard)</td>
<td>1:1</td>
</tr>
<tr>
<td>% of Lupeol</td>
<td>6.85% w/v</td>
</tr>
</tbody>
</table>

DISCUSSION AND CONCLUSION

Lupeol has been found to play a vital role when obtained from different plant extracts reported in various studies. Our research studies draw the readers’ vision towards the constituent in the petroleum ether Benincasa hispida seed extract confirmed by TLC, HPLC and HPTLC. The current research studies urge the young scientists to note the strong pharmacological prospects and correlate the same by undertaking studies on pharmacological models of different therapeutic categories.

ACKNOWLEDGEMENT

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